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The Regulation of Proopiomelanocortin Gene Expression by Immune System Peptides

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Viral or bacterial infection challenges the immune system, one of the primary responses being an alteration in the circulating levels of immune cytokines. These cytokines play a role in coordinating the cellular immune response. More recently, it has been hypothesized that cytokines also have potential effects on cells outside the immune system (such as those in the central nervous system) which may ultimately feed back to affect the response of the immune system. One of the important responses may be in cells which express the neuropeptide and stress-related genes, proopiomelanocortin (POMC), proenkephalin, and prodynorphin. These genes encode peptides essential to the stress response and function of the CNS including ACTH, β-endorphin, enkephalin, and dynorphin. Recently, we have demonstrated a very high level of expression of the proenkephalin gene in glial cells of the CNS, specifically astrocytes. Furthermore, we have found that cytokines such as tumor necrosis factor-α (TNF α) and interleukin 1 stimulate proenkephalin expression while γ-interferon inhibits expression. These results suggest that cytokines regulate the expression of a glial cell opioid gene, which in turn may dramatically influence neural functioning and the capacity to respond to the stress of infection.

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INTRODUCTION

Neuroimmune interactions can occur through a variety of mechanisms and in the central nervous system there exists increasing evidence that glial cells may serve as important mediators of these interactions (1). The interactions between glial cells, neurons, and the immune system have all been previously demonstrated. The initiation of glial-neuronal interactions occurs at an early point during central nervous system development where glial cells can form the basis for directing neuronal outgrowth and network formation (2, 3) and neurons can potentially influence glial progenitor cell development (4, 5). These interactions continue throughout development and also involve cell-to-cell communication via soluble factors such as neurotransmitters and neuropeptides. Astrocytes have previously been demonstrated to express a number of receptors for excitatory and inhibitory amino acids (6, 7, 8) and neurotransmitters (9, 10, 11, 12). Additionally, a number of these excitatory amino acids and neurotransmitters have been demonstrated to be capable of evoking intracellular responses within astrocytes and regulating astrocytic function. For example, glutamate and β-adrenergic agonists can induce fluxes in Ca^{2+} (7) and cAMP (9, 10, 12) levels in astrocytes respectively. More recently astrocytes have been demonstrated to express a number of neuropeptide-encoding genes such as the proenkephalin and somatostatin genes (13, 14, 15, 16, 17). These observations suggest that neurons and astrocytes communicate much more actively than previously realized and that the bi-directional interaction allows the close regulation of either glial or neuronal function by neurons or glia respectively.

Glial cells within the central nervous system represent important and complex interfaces with the peripheral immune system. Astrocytes are hypothesized to induce the formation and contribute towards the maintainence of the blood-brain barrier (18, 19, 20) and microglia are apparently developmentally derived from peripheral immune tissues such as bone marrow (21). Both astrocytes and microglia represent the APCs within the central nervous system and have

previously been demonstrated to express class I and II MHC antigens (1, 21, 22, 23). In addition, astrocytes are capable of expressing cytokines including IL-1 (24, 25), IL-3 (26), IL-6 (25), α/β-IFNs (27), TNF-α (25, 28), and hemopoietic CSFs such as granulocyte-CSF and granulocyte/macrophage CSF (29, 30). Some of these cytokines have been found to regulate astrocytic function such as the induction of class I and II MHCs by γ-IFN (31, 32, 33) and the stimulation of astrocytic proliferation by both IL-1 (34, 35) and TNF-α (36).

Reactive gliosis is the term applied to the rapid proliferation of astrocytes surrounding the site of central nervous system injury or trauma (18). This rapid increase in astrocyte number and size contributes to the formation of the gliotic scar which was formerly thought to impede the restoration of neuronal networks. Recent evidence suggests that reactive astrocytes may actually function during early gliosis to restore disrupted neuronal networks by the secretion of neurotrophic factors such as nerve growth factor (37, 38) and ciliary neurotrophic factor (39, 40). Many in vitro models of gliosis such as the cAMP-induced changes in cultured astrocyte glial fibrillary acidic protein content, vimentin content and morphology indicate remarkable similarities with reactive astrocytes in vivo (41). We have previously demonstrated that the cAMP-induced increases in glial fibrillary acidic protein mRNA levels in primary cultures of neonatal rat astrocytes is correlated with a dramatic increase in proenkephalin gene expression (15). Since the levels of several cytokines such as IL-1 (34) and TNF-α (42) increases rapidly during instances of reactive gliosis in vivo such as after brain injury or disease, we were interested in determining if these cytokines regulate proenkephalin gene expression in primary cultures of astrocytes.

MATERIALS AND METHODS

Animals, Tissues and Materials

Untimed pregnant Sprague-Dawley rats were obtained from Bantin and Kingman

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(Fremont, CA) and kept under a 14 hour light, 10 hour dark regimen with food and water available ad libitum. The pregnancies were carried to full term and the neonatal rat pups (1-2 days post-natal) were used for the isolation of primary cultures of astrocytes. The cytokines used in the experiments were recombinant forms of human IL-1β (Genzyme, Boston, MA), human TNF-α (Genentech, South San Francisco, CA) and rat γ-IFN (Amgen, Thousand Oaks, CA). Unless otherwise stated, all reagents used were Molecular Biology grade (Sigma, St. Louis, MO).

Isolation and Culture of Primary Cultures of Neonatal Rat Astrocytes

Primary cultures of neonatal rat astrocytes were isolated and cultured as previously described (15) using the method of McCarthy and de Vellis (16). Briefly, neonatal rat pups (1-2 day post-natal) were sacrificed by decapitation, the cerebral cortices were quickly removed under aseptic conditions and the meninges dissected out to remove fibroblast contamination. The brain cells were mechanically dispersed in Dulbecco's Modified Eagle Media:Nutrient Mixture F-12 (Ham) containing 3.2 g/L D-glucose, 0.365 g/L L-glutamine, 15 mM HEPES, 8.1 mg/L phenol red and 55 mg/L sodium pyruvate (GIBCO Laboratories, Grand Island, NY) supplemented with 10% (v:v) fetal bovine serum (Hyclone, Logan, UT) and filtered consecutively through 230 mm and 130 mm membranes (Tetko, Elmsford, NY). The brain cells were cultured for 7-8 days in Dulbecco's Modified Eagle Media:Nutrient Mixture F-12 (Ham) supplemented with 10% (v:v) fetal bovine serum after which overlying phase-dark bipotential glial precursor cells (termed mixed oligodendrocyte-type-2-astrocyte lineage cells) were shaken off at 200 cycles/minute for 18-24 hours and the remaining type I astrocyte cell population were reshaken for an additional 18 hours to remove possible microglial contamination. The astrocytes were then reseeded into 225 cm2 flasks and cultured for 6-7 days until confluent. Representative populations of type I astrocytes prepared in this way are routinely 98-99% homogeneous by positive immunohistochemical staining using antisera against glial fibrillary acidic protein and infrequent tests have proven negative for Mac-1, myelin basic protein and glycerol phosphate dehydrogenase.

Cytokine Treatments

In the experiments represented in figures 1, 2 and 3, cultures of astrocytes were either untreated for 48 hours (Controls), treated with 50 ng/ml of TNF-α for the last 24 hours of the total 48 hour treatment period (TNF-a), treated with 20 u/ml of g-IFN for the total 48 hour treatment period (γ-IFN) or a combination of 20 u/ml of γ-IFN for 48 hours and 50 ng/ml of TNF-α for the last 24 hours of the total 48 hour treatment period (TNF-α/γ-IFN). In the experiments represented in figure 4, cultures of astrocytes were either untreated for 24 hours (Controls) or treated with 10 ng/ml of IL-1β for 24 hours (IL-1β). A similar treatment regimen was previously used to demonstrate maximal induction of class II MHC antigen expression in astrocytes (22).

RNA Isolation and Northern Analysis

After the cytokine treatments, the cultures of astrocytes were rinsed in PBS, lysed and homogenized in the presence of 4.0 M guanidine thiocyanate solution and total RNA was isolated by cesium chloride ultracentrifugation as previously described (44). RNA pellets were resuspended in 5 mM EDTA pH 8.0, extracted once with an equal volume of 1:1 phenol/chloroform, twice with equal volumes of 24:1 chloroform/isoamyl alcohol and then ethanol precipitated in 2.5 volumes of ethanol at -20°C overnight. RNA content was determined spectrophotometrically at 260 nm. Equal amounts of RNA samples were then denatured in 2.2 M formaldehyde and 50% (v/v) formamide at 55°C for 15 minutes before size-separation through 1.5% (w/v) agarose gels under denaturing conditions (2.2 M formaldehyde), transfered onto Nytran membranes (Schleicher and Schuell, Keene, NH) by capillary action with 20 x SSC (1 x SSC = 0.15 M NaCl, 15 mM trisodium citrate) and fixed by baking at 80°C for 2 hours. Blots were prehybridized with 0.25 M NaCl, 0.25 M Na_2HPO_4, 1 mM EDTA, 7% (w/v) SDS, and 1% (w/v) BSA (Prehybridization buffer) at 45°C for 18 hours before hybridization with 50 mCi of ^{32}P-labeled cDNA probe per 100 ml of Hybridization buffer (essentially Prehybridization buffer containing 50% (v/v) formamide) at 45°C for 48 hours. Proenkephalin transcripts were detected using a ^{32}P-labeled 1.1 kb Eco R1/Hind III fragment of the rat proenkephalin cDNA (45) and glutamine synthetase transcripts were detected using a ^{32}P-labeled 2.4 kb Eco R1 fragment of the rat glutamine synthetase cDNA (46). The DNA fragments were radiolabeled according to the method of Feinberg and Vogelstein (47, 48). After hybridization,

the blots were washed once with 5 x SSC, 1% (w/v) SDS at 45°C for 30 minutes, twice with 2 x SSC, 0.1% (w/v) SDS at 45°C for 30 minutes each and once with 0.1 x SSC, 0.1% (w/v) SDS at 60°C for 15 minutes. Autoradiography was performed at -70°C with intensifying screens. The blots were stripped of probe by a wash with 5 mM Tris/HCl pH 8.0, 0.2 mM EDTA, 0.05% (w/v) pyrophosphate, 0.1% (w/v) SDS and 0.1 x Denhardt's (1 x Denhardt's = 0.02% (w/v) each of Ficoll, BSA and polyvinyl-pyrrolidone) at 70°C for 2 hours before rehybridization. The autoradiographic signals were quantified by laser densitometric scans using an LKB Ultroscan.

Radioimmunoassay for Proenkephalin-derived Peptides

Media from primary cultures of astrocytes after the different cytokine treatments were assayed for proenkephalin-derived peptides by radioimmunoassay essentially as previously described using solid-phase synthesized met-enkephalin-arg^6-phe^7 as a standard and specific antisera against met-enkephalin-arg^6-phe^7 (49, 50). Briefly, the assay was modified to use dextran-coated charcoal to separate bound from free and in this form the assay has a sensitivity of 20 pg and recognizes free met-enkephalin-arg^6-phe^7, intact proenkephalin as well as any proenkephalin-derived peptides which contain the heptapeptide sequence in its carboxyl terminus. Media samples from individual culture flasks were assayed following extraction. The extraction procedure consisted of the acidification of media samples to 0.1% (v/v) trifluoroacetic acid and application to WP Butyl (C4) disposable extraction columns (J.T. Baker, Phillipsburg, NJ). The preparation of the columns has been previously described (51). Briefly, after washing each column with 5 ml of 0.1% (v/v) trifluoroacetic acid, peptides were eluted with 5 ml of 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid. The eluate was diluted twice with H_2O, frozen to -70°C and lyophilized. The lyophilized extract was then resuspended in 13.6% (v/v) acetonitrile/0.1% (v/v) trifluoroacetic acid assayed for met-enkephalin-arg^6-phe^7 as described above.

RESULTS

Regulation of astrocyte gene expression by TNF-α and γ-IFN

The treatment of primary cultures of neonatal rat cerebral astrocytes with 50 ng/ml TNF-α (for the last 24 hours of a total 48 hour treatment period) increased proenkephalin mRNA levels by almost two-fold relative to the untreated controls (Figure 1a, Control versus TNF-α). In contrast, treatment with 20 U/ml γ-IFN (for the total 48 hour treatment period) decreased proenkephalin mRNA levels by 50% relative to the untreated controls (Figure 1a, Control versus γ-IFN).

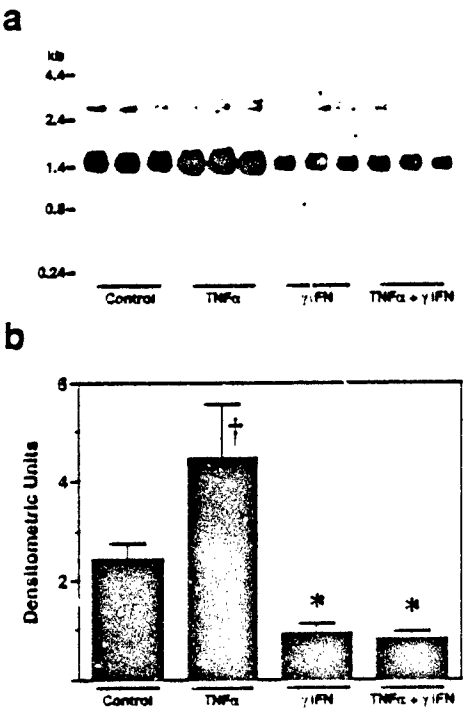

Figure 1. Northern blot analysis of total RNA isolated from primary cultures of neonatal rat cerebral astrocytes treated with TNF-α and γ-IFN and probed for proenkephalin mRNA. (a) Primary cultures of neonatal rat cerebral astrocytes were isolated, treated with 50 ng/ml TNF-α and 20 U/ml γ-IFN for 24 and 48 hours respectively. The TNF-a treatment was for the second 24 hours of the treatment period. Each lane represents 60 mg of total RNA which has been size-separated

through denaturing agarose electrophoresis, transferred onto Nytran by blotting, fixed by baking and proenkephalin transcripts were detected as described in the Materials and Methods. (b) Histogram representing the laser densitometric scan of the autoradiograph in (a). Data are represented as the mean ± SEM.†, Statistically significant difference compared to the Control value (Unpaired Student's t test, $P < 0.04$, $n = 6$).*, Statistically significant difference compared to the Control value (Unpaired Student's t test, $P < 0.002$, $n = 3$).

Interestingly, treatment with 50 ng/ml TNF-α (for the last 24 hours of a total 48 hour treatment period) was unable to reverse the inhibitory effect of 20 u/ml γ-IFN pretreatment (for the total 48 hour treatment period)(Figure 1a, γ-IFN versus TNF-α/γ-IFN). Thus the treatment of primary cultures of neonatal rat cerebral astrocytes with the cytokines TNF-α and γ-IFN resulted in statistically significant increases (Figure 1b, †, unpaired Student's t test, $P < 0.04$, $n = 6$) and decreases (Figure 1b, *, unpaired Student's t test, $P < 0.002$, $n = 3$) in proenkephalin mRNA levels respectively. We previously found that proenkephalin mRNA levels in primary cultures of neonatal rat cerebral astrocytes were decreased with γ-IFN but unaffected by TNF-α (52). However, when we corrected for the amount of total RNA that was present in each lane in the Northern analysis, we found that although the inhibitory effect of γ-IFN remained statistically significant, the levels of proenkephalin mRNA were significantly increased by TNF-α.

In contrast, glutamine synthetase mRNA levels in these primary cultures of neonatal rat cerebral astrocytes did not appear to be changed significantly by treatment with TNF-α, γ-IFN or a combination of both TNF-α/γ-IFN (Figure 2, cytokine treatments not statistically different from untreated controls, unpaired Student's t test). The identical blot that was used to generate the Northern analysis data in figure 1 was used to generate the Northern analysis data in figure 2.

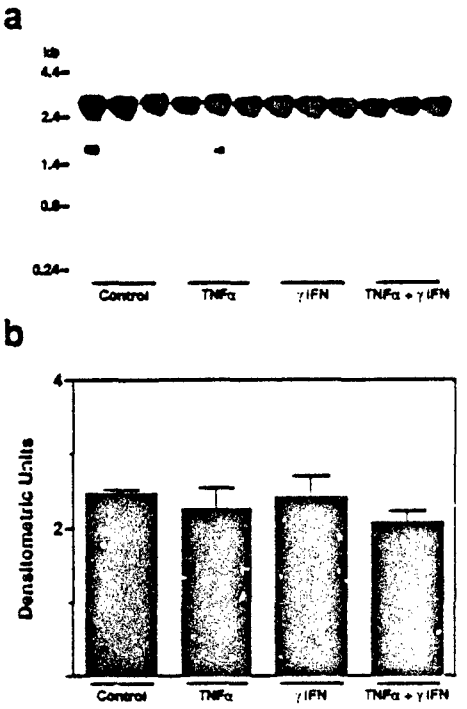

Figure 2. Northern blot analysis of total RNA isolated from primary cultures of neonatal rat cerebral astrocytes treated with TNF-α and γ-IFN and probed for glutamine synthetase mRNA. (a) The identical blot used to generate the autoradiograph in Figure 1a was stripped of probe and glutamine synthetase transcripts were detected using a ^{32}P-labeled rat cDNA. (b) Histogram representing the laser densitometric scan of the autoradiograph in (a). Data are represented as the mean ± SEM. None of the different cytokine treatments revealed statistically significant differences in the levels of glutamine synthetase transcripts when compared to the Control value (Unpaired Student's t test).

RIA for met-enkephalin-arg^6-phe^7 in the media of primary cultures of neonatal rat cerebral astrocytes revealed that the effect of the cytokines TNF-α and γ-IFN on proenkephalin mRNA levels was translated to effects on proenkephalin-derived peptides secreted by the astrocytes. The treatment of primary cultures of neonatal rat cerebral astrocytes with 50 ng/ml TNF-α (for the last 24 hours of a total 48 hour treatment period) increased the amount of immunoreactive met-enkephalin-arg^6-phe^7 by almost two-fold relative to the untreated controls (Figure 3, CON versus TNF-α). In contrast, treatment with 20 U/ml γ-IFN (for the total 48 hour treatment period) decreased the amount of immunoreactive met-enkephalin-arg^6-phe^7 by almost 50% relative to the untreated controls (Figure 3, CON versus γ-IFN). Interestingly, treatment with 50 ng/ml TNF-α (for the last 24 hours of a total 48 hour treatment period) was unable to reverse the inhibitory effect of 20 u/ml γ-IFN pretreatment (for the total 48 hour treatment period)(Figure 3, γ-IFN versus TNF-α/γ-IFN). Thus, the treatment of primary cultures of neonatal rat cerebral astrocytes with the cytokines TNF-α and γ-IFN resulted in statistically significant increases (Figure 3, †, unpaired Student's t test, $P < 0.02$, $n = 3$) and decreases (Figure 3, *, unpaired Student's t test, $P < 0.002$, $n = 3$) in the amounts of secreted proenkephalin-derived peptides respectively.

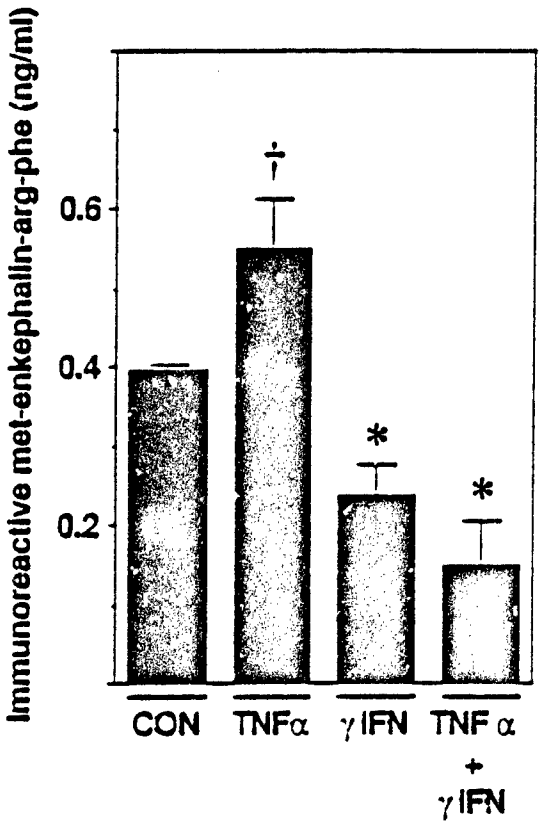

Figure 3. Immunoreactive met-enkephalin-arg^6-phe^7 in culture media of primary cultures of neonatal rat cerebral astrocytes treated with TNF-α and γ-IFN. Primary cultures of neonatal rat cerebral astrocytes were isolated and treated with TNF-α and γ-IFN as described in Figure 1. Culture media was analyzed for ir-MERF by RIA using an antisera recognizing intact proenekphalin. Data are represented as the mean ± SEM. †, Statistically significant difference compared to the Control (CON) value (Unpaired Student's t test, $P < 0.02$, $n = 3$).*, Statistically significant difference compared to the Control value (Unpaired Student's t test, $P < 0.002$, $n = 3$).

Regulation of astrocyte proenkephalin mRNA levels by IL-1β

The treatment of primary cultures of neonatal rat cerebral astrocytes with 10 ng/ml of IL-1β for 24 hours increased the levels of proenkephalin mRNA by almost two-fold relative to untreated controls (Figure 4a, lanes 1 and 3 versus lanes 2 and 4). The effects of IL-1β on proenkephalin gene expression in the primary cultures of neonatal rat cerebral astrocytes was determined to be statistically significant (Figure 4b, unpaired Student's t test, $P < 0.03$, $n = 3$).

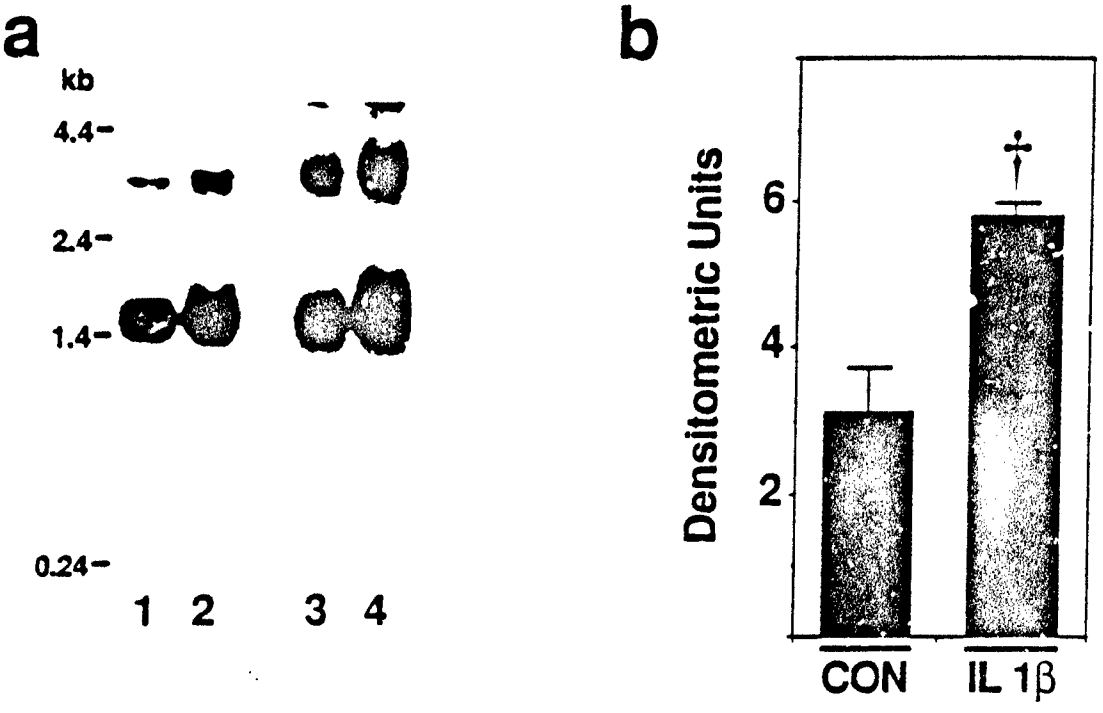

Figure 4. Northern blot analysis of total RNA isolated from primary cultures of neonatal rat cerebral astrocytes treated with IL-1β and probed for proenkephalin mRNA.

(a) Primary cultures of neonatal rat cerebral astrocytes were isolated, treated with 10 ng/ml IL-1β for 24 hours and total RNA isolated as described in the Materials and Methods. Each lane represents 60 mg of total RNA which has been size-separated through denaturing agarose electrophoresis, transfered onto Nytran by blotting, fixed by baking and proenkephalin transcripts were detected as described in the Materials and Method Lanes 1 and 2 were from the first experiment and lanes 3 and 4 were from a second experiment. Controls : Lanes 1 and 3, IL-1β : Lanes 2 and 4.

(b) Histogram representing the laser densitometric scan of the autoradiograph in (a). Data are represented as the mean ± SEM.†, Statistically significant difference compared to the Control value (Unpaired Student's t test, $P < 0.03$, $n = 3$).

DISCUSSION

Proenkephalin gene expression in primary cultures of neonatal rat cerebral astrocytes is increased by treatment with both of the cytokines TNF-α and IL-1β but decreased by treatment with γ-IFN. In contrast, glutamine synthetase gene expression is not changed significantly by either TNF-α or γ-IFN suggesting that the effects of the cytokines are specific for proenkephalin gene expression and not a generalized effect on gene expression in astrocytes. It is also unlikely that the decreases in proenkephalin gene expression are due to the cytotoxic effects of γ-IFN.

The effects of the cytokines TNF-α and γ-IFN are observed to be on the levels of proenkephalin mRNA as well as on the amount of proenkephalin-derived peptides secreted by the astrocytes. The changes observed in proenkephalin mRNA levels can be attributed to either a change in the rate of gene transcription or transcript stability, however, these studies do not however definitively differentiate between the possibilities. Interestingly, a visual search of the 5' flanking region of available sequences for the rat proenkephalin gene (53) revealed a region from -40 to -52, GTTTGGCTTCTCC, which contains 10 out of 13 base pairs identical to IFN-response motifs present in the upstream elements of IFN-regulated genes (54). This suggests that the effects of γ-IFN may be exerted at least in part through the regulation of gene transcription. It is interesting to note that in contrast to the inhibitory effects of γ-IFN on proenkephalin expression, most of the other effects of γ-IFN on gene expression in astrocytes appear to be stimulatory, such as the effects on class I and II MHC gene induction (31, 32, 33). The dramatically divergent effects of TNF-α and γ-IFN on proenkephalin gene expression in astrocytes are surprising considering previous studies demonstrating that these cytokines act synergistically to induce class II antigen expression in astrocytes and that this occurs through the stimulation of TNF-α receptor number by γ-IFN (22). All of these observations suggest that the effect of γ-IFN on proenkephalin gene expression in astrocytes is mediated mainly through a pathway independent of TNF-α receptor induction and that the potential exists for the effects to be mediated through a putative IFN-inducible enhancer element.

The effects of TNF-α and γ-IFN on the secretion of proenkephalin-derived peptides by astrocytes suggest that these cytokines may also have direct effects on stimulating the release of proenkephalin-derived peptides. However, it is interesting to note that astrocytes do not appear to possess electron-dense core secretory granules (16) and that the predominant form of

proenkephalin-derived peptide secreted by astrocytes is the intact precursor, primarily associated with intermediate filaments (15, 16). This suggests that the intact proenkephalin precursor might not be secreted through the classical regulated pathway of secretion but either through the constitutive pathway of secretion or a novel alternative pathway.

A number of previous studies have demonstrated that proenkephalin gene expression appears to be correlated with rapidly proliferating cells and the undifferentiated state (55, 56, 57, 58, 59, 60). The levels of the cytokines TNF-α and IL-1β increase rapidly during reactive gliosis in vivo after brain injury and disease and these cytokines have also previously been demonstrated to stimulate the rapid proliferation of astrocytes (34, 35, 36). Thus, it is not surprising that treatment with the cytokines TNF-α and IL-1β increases proenkephalin gene expression in astrocytes. It is interesting to note that a visual search of the available 5' flanking region of the rat proenkephalin gene (53) did not reveal the presence of any DNA elements homologous to the NF kB enhancer which is thought to mediate the effects of TNF-α and IL-1 in the activation of the IL-6 gene and the HIV enhancer (61, 62). However this does not exclude the possibility that TNF-α and IL-1β may act through NF kB enhancer elements to increase proenkephalin gene expression as there may be further DNA elements corresponding to the NF kB enhancer located upstream to the -450 bp available 5' flanking sequence information.

This demonstration that cytokines have the potential to regulate neuropeptide gene expression in a specific glial cell type in vitro suggests that glial cells may serve an important and pivotal role in mediating the interactions between the immune and the neural systems during central nervous system diseases such as multiple sclerosis (42) or after central nervous system trauma (34) or infection (63) which are accompanied by a rapid proliferation of astrocytes. The onset of HIV encephalopathy after HIV infection may be also be mediated through glial cells. Recent studies have suggested specific tropism of HIV-1 for microglia (64) however, this does not exclude possible roles for astrocytes in mediating neuroimmune communication after HIV infection through microglial-derived cytokines.

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